REFERENCES

1. T. B. Turner, E. Mezey and A. W. Kimbal, *John Hopkins med. J.* **141**, 235 (1977).
2. Y. Israel, L. Videla and J. Bernstein, *Fedn. Proc.* **34**, 2052 (1975).
3. N. R. Di Luzio and T. E. Stege, in *Alcohol and the Liver* (Eds. M. M. Fisher and J. G. Rankin), p. 45. Plenum Press, New York (1977).
4. A. I. Cederbaum, *Mt. Sinai J. Med.* **47**, 317 (1980).
5. L. A. Videla and A. Valenzuela, *Life Sci.* **31**, 2395 (1982).
6. L. A. Videla, V. Fernández, G. Ugarte and A. Valenzuela, *Fedn. Eur. Biochem. Soc. Lett.* **111**, 6 (1980).
7. C. Guerri and S. Grisolia, *Pharmac. Biochem. Behav.* **13**, 53 (1980).
8. C. Hetu, L. Yelle and H-G. Joly, *Drug Metab. Dispos.* **10**, 246 (1982).
9. N. S. Kosower and E. M. Kosower, *Int. Rev. Cytol.* **54**, 109 (1979).
10. A. Meister, in *Metabolic Pathways* (Ed. D. M. Greenberg), Vol. 7, p. 101. Academic Press, New York (1975).
11. B. Chance, H. Sies and A. Boveris, *Physiol. Rev.* **59**, 527 (1979).
12. V. Koster, D. Albrecht and H. Kappus, *Toxic. appl. Pharmac.* **41**, 639 (1977).
13. L. A. Videla, C. G. Fraga, O. R. Koch and A. Boveris, *Biochem. Pharmac.* **32**, 2822 (1983).
14. L. A. Videla, H. Iturriaga, D. Bunout, A. Valenzuela, M. E. Pino and G. Ugarte, *Clin. Sci.* **66**, 283 (1984).
15. T. Suematsu, T. Matsumura, N. Sato, N. Miyamoto, T. Ooka, T. Kamada and H. Abe, *Alcoholism: Clin. expl. Res.* **5**, 427 (1981).
16. A. Valenzuela, V. Fernández and L. A. Videla, *Toxic. appl. Pharmac.* **70**, 87 (1983).
17. J. M. Martinez, F. Sánchez, R. Calvo, A. Garrido and J. Merchante, in *New Trends in the Therapy of Liver Diseases* (Ed. A. Bertelli), p. 201. Karger, Basel (1975).
18. L. A. Videla, V. Fernández, A. Valenzuela and G. Ugarte, *Pharmacology* **22**, 343 (1981).
19. T. F. Slater and M. N. Eakins, in *New Trends in the Therapy of Liver Diseases* (Ed. A. Bertelli), p. 84. Karger, Basel (1975).
20. L. A. Videla, *Experientia* **39**, 500 (1983).
21. A. I. Cederbaum and G. Cohen, *Subst. Alc. Actions/Misuse* **2**, 269 (1981).
22. B. Janiak and R. Hansel, *Planta med.* **8**, 71 (1960).
23. P. Platt and B. Schenorr, *Arzneimittel-Forsch.* **21**, 3637 (1971).
24. G. Hahn, *Arzneimittel-Forsch.* **18**, 698 (1968).
25. F. Medina, *Gazz. med. ital.* **136**, 1 (1977).
26. G. P. Lewis and P. J. Pipper, *Nature, Lond.* **254**, 308 (1975).
27. G. Vogel, *Arzneimittel-Forsch.* **18**, 1063 (1968).
28. H. M. Raven and H. Schriewer, *Arzneimittel-Forsch.* **21**, 1194 (1971).
29. G. Vogel and S. Tomme, *Arzneimittel-Forsch.* **19**, 613 (1969).
30. H. Schriewer, R. Badde, G. Roth and H. M. Raven, *Arzneimittel-Forsch.* **23**, 157 (1973).
31. A. Greimel and H. Koch, *Experientia* **33**, 1417 (1977).
32. A. Bindoli, L. Cavallini and N. Siliprandi, *Biochem. Pharmac.* **26**, 2405 (1977).
33. E. Bernt and H. U. Bergmeyer, in *Methods for Enzymatic Analysis* (Ed. H. U. Bergmeyer), Vol. 4, p. 1643. Academic Press, New York (1974).
34. K. M. Wilbur, F. Bernhein and O. W. Shapiro, *Archs. Biochem. Biophys.* **29**, 305 (1949).
35. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
36. T. Asakawat and S. Matsushica, *Lipids* **14**, 401 (1979).
37. A. Boveris, A. Cadenas and B. Chance, *Fedn. Proc.* **40**, 195 (1981).
38. L. A. Videla, V. Fernández, N. Fernández and A. Valenzuela, *Subst. Alc. Actions/Misuse* **2**, 153 (1981).
39. G. W. Winston and A. I. Cederbaum, *Pharmac. Biochem. Behav.* **18**, 189 (1983).

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The inhibition of bovine liver dihydrofolate reductase by tricyclic antidepressant drugs

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The inhibition of dihydrofolate reductase (5,6,7,8-tetrahydrofolate NADP oxidoreductase EC 1.5.1.3) by folic acid analogues has been well documented [1, 2]. The tricyclic anti-depressant drugs possess little structural similarity to these folate-analogue inhibitors and would not be expected to inhibit dihydrofolate reductase. The present study, however, reports significant inhibition of the bovine liver enzyme by such drugs. Six tricyclic drugs were studied; amitriptyline, butriptyline, desmethylnortriptyline, imipramine, nortriptyline and protriptyline.

Materials and methods

Materials. Folic acid and bovine liver dihydrofolate reductase were purchased from Sigma Chemical Co. Ltd. (Poole, Dorset, U.K.). Concentrated hydrochloric acid was from Fisons Scientific Apparatus (Loughborough, Leicestershire). Reduced NADP was obtained from Cambrian Chemicals (Croydon, U.K.). All other chemicals were from British Drug Houses Chemicals Ltd. (Poole, Dorset, U.K.). All reagents were of the highest grade or purity available.

Synthesis of dihydrofolate. The method employed was that of Wilmanns [3] and involved reduction of folic acid in the presence of ascorbic acid and sodium dithionite. The unstable dihydrofolate was synthesised prior to each experiment. The crystals obtained were suspended in 5 mM hydrochloric acid and kept in the dark at 0°. Concentrations of dihydrofolate were determined using the published molar absorbance coefficient of a solution of dihydrofolate in 10 mM potassium hydroxide ($= 22 \times 10^3$ l/mole/cm at 283 nm).

Enzyme assays. The method used was based on that of Wilmanns [3]. In a final reaction volume of 0.5 ml the following components were present: citrate buffer (0.1 M pH 5.4), 2-mercaptoethanol (12.5 mM), NADPH (varied in five stages from 0.04 to 1 mM), dihydrofolate (DHF) (varied in five stages from 1.4 to 34.7 mM) and dihydrofolate reductase (13 mIU/ml). Where appropriate, the tricyclic drug under investigation was present in the final reaction mixture at four concentration levels in the range of 4–200 μM. In a control experiment the inhibition of the enzyme by methotrexate (amethopterin, 4-amino-

N^{10}-methylfolic acid) was investigated under the same reaction conditions. The methotrexate was present in a final reaction concentration in the range 0.2–20 nM.

All reactions were initiated by the addition of the enzyme to an otherwise complete reaction mixture. Absorbance measurements at 340 nm were taken automatically every 10 sec during the first minute of the reaction using a Cecil CE 292 Digital Spectrophotometer fitted with micro-sipette and digital printer. All reactions were carried out in duplicate at 30° and the mean of the duplicate observation was taken to be a measure of the initial velocity (v_i) of the reaction.

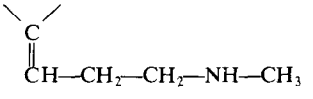
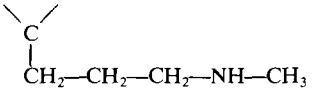
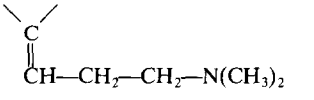
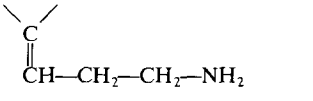
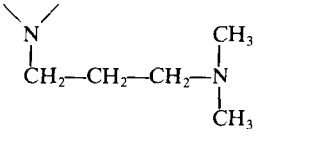
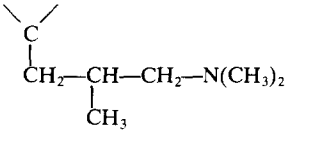
Treatment of results. All results were examined by both manual and computer methods. The manual determination of the kinetic constants involved the plotting of $1/v_i$ against reciprocal substrate concentration in the manner of Lineweaver and Burk [4]. The computer program employed was the Fortran program described by Cornish-Bowden and Endreny [5] which is a robust weighted regression program for fitting equations to kinetic enzyme data which requires minimal assumptions about the error structure of that data. The dependence of the variances on the velocities is not assumed but is deduced from internal evidence in the data itself.

Results and discussion

Table 1 shows the inhibition constants (K_i calculated for the tricyclic drugs examined. For methotrexate the K_i values were less than 25 nM when NADPH and DHF were varied. The investigation revealed inhibition of bovine liver dihydrofolate reductase by five of the six tricyclic drugs examined. Butriptyline did not cause significant inhibition at 200 μM. Nortriptyline was the most potent inhibitor with a K_i of 45 μM (NADPH varied) and 70 μM (dihydrofolate varied). Thus small changes in the structure cause considerable variation in K_i.

A common structural feature of these drugs is a side chain of three carbon atoms linked to a nitrogen. This can be compared to the C(7)–C(6)–C(9)–N(10) portion of folic acid. In the case of nortriptyline, protriptyline and amitriptyline the methyl groups attached to the nitrogen could aid binding whereas desmethylnortriptyline lacks any group comparable to the benzoic acid group in folic acid. With butriptyline the additional methyl group on the side chain

Table 1. The inhibition of dihydrofolate reductase by tricyclic drugs

Drug	Structure of side chain	K_i Values† (μM) NADPH Varied	K_i Values† (μM) DHF Varied
Nortriptyline		45 (±15)	70 (±17)
Protriptyline*		61 (±11)	86 (±23)
Amitriptyline		146 (±28)	52 (±24.4)
Desmethylnortriptyline		249 (±109)	240 (±101)
Imipramine		646 (±313)	330 (±132)
Butriptyline		No inhibition	No inhibition

* Protriptyline has a double bond in the ring between carbon atoms 5 and 6.
† Standard deviations shown in parentheses.

could cause the reduced binding. In imipramine a ring nitrogen replaces the carbon found in amitriptyline and a double bond, which is present in folic acid, is lost. These changes could account for the increase in K_i values.

Thus a new class of compounds, the tricyclic antidepressant drugs, cause significant inhibition of dihydrofolate reductase. In considering the practical implications of this finding it should be borne in mind that the K_i values are more than 1000-fold greater than that for methotrexate while the therapeutic blood levels used for the tricyclic drugs are about 100-fold lower than those used for methotrexate. Nevertheless, it is possible that long term treatment with the tricyclic antidepressant drugs could lead to their accumulation in tissues—the consequences of this in terms of exacerbation of folate deficiency require further consideration.

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REFERENCES

1. J. W. Williams, R. G. Duggleby, R. Culter and J. F. Morrison, *Biochem. Pharmac.* **29**, 589 (1980).
2. J. C. White, *J. biol. Chem.* **254**, 10889 (1979).
3. W. Wilmanns, in *Methods of Enzymatic Analysis* (Ed. H. U. Bergmeyer), Second English Edition, p. 666. Academic Press, London (1974).
4. H. Lineweaver and D. Burk, *J. Am. chem. Soc.* **56**, 658 (1934).
5. A. Cornish-Bowden and L. Endrenyi, *Biochem. J.* **193**, 1005 (1981).

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Characterization of the interactions of gallamine with muscarinic receptors from brain

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A previous report by Ellis and Hoss [1] demonstrated that gallamine inhibits the binding of the potent and specific muscarinic antagonist quinuclidinyl benzilate (QNB). Although other studies have found gallamine to be an antagonist in muscarinic systems [2–5], the binding properties of gallamine were shown to be similar to those of muscarinic agonists. That is, the occupancy curve for gallamine is better suited to a model of two populations of receptors than to a one-site model and it possesses greater overall affinity toward brainstem receptors than toward those of the forebrain [1]. Recently, Stockton *et al.* [6] reported that gallamine regulates muscarinic receptors by an allosteric mechanism. The possibility was therefore raised that the heterogeneity detected by gallamine in the earlier study might have been artifactual, due to the assumption of an incorrect mechanism of action. However, it is difficult to account for all of the previous findings of Ellis and Hoss by a noncompetitive mechanism, especially that the elimination of receptors with low affinity for the agonist carbachol results in a loss of receptors with low affinity for gallamine [1]. The present study was undertaken in an attempt to reconcile these apparently discrepant reports and to further evaluate the mechanism(s) by which gallamine interacts with muscarinic receptors.

Tritiated L-QNB (32.2 Ci/mmole) and [^{3}H]*N*-methylscopolamine (NMS, 84.8 Ci/mmole) were obtained from the New England Nuclear Corp. (Boston, MA). Gallamine triethiodide was obtained from K & K Laboratories (Plainsview, NY). Neural membranes were prepared from the forebrains of male Sprague–Dawley rats as described previously [1] and stored frozen at −70° until used. The term forebrain denotes the portion of the brain anterior to the diencephalon, plus overlying cortical and hippocampal tissue. Binding assays were conducted at 25° in 40 mM sodium–potassium phosphate buffer (except where otherwise indicated), pH 7.2, and were terminated by filtration through GF/B glass fiber filters (Brandel, Gaithersburg, MD). In all cases, nonspecific binding was determined by the inclusion of 1 μM unlabeled QNB with the labeled ligand. Under conditions of low ionic strength, high concentrations of gallamine were found to decrease the nonspecific binding of [^{3}H]QNB. Therefore, nonspecific binding was determined for each concentration of gallamine by including 1 μM unlabeled QNB with the labeled QNB and gallamine in a separate set of assay tubes.

The results of experiments which employed [^{3}H]NMS (Figs. 1A and 2A) were consistent with the report by Stockton *et al.* [6]. Both the association and dissociation kinetics were dramatically slowed by the presence of gallamine. Separate studies (data not shown) found the half-maximal effect on the off-rate of [^{3}H]NMS to occur at 30 μM gallamine. However, when similar studies were carried out with [^{3}H]QNB (Figs. 1B, 1C, and 2B), no allosteric effects were noted. Even at 100 μM, gallamine failed to significantly alter the rate of association of [^{3}H]QNB or the rate of dissociation of previously bound [^{3}H]QNB.

The next question that we wished to address was whether the muscarinic sites that appear to possess different affinities for gallamine are separate and non-interconvertible. One approach to this question is to selectively block the low-affinity sites with an irreversible or slowly reversible antagonist (e.g. QNB), as has been done in the past for the sites that are differentiated by the muscarinic agonists [1, 8, 9]. Initial studies (Fig. 3) found that the different sites expressed the most widely disparate affinities for gallamine under conditions of low ionic strength. Such conditions were therefore chosen for the protection experiments. Forebrain membranes were preincubated in the presence of unlabeled QNB and a concentration of gallamine that would compete efficiently only at gallamine's high-affinity sites. Extensive washing then removed bound and unbound gallamine and unbound QNB; more than 90% of the bound